

Synergistic Interactions of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in an *In Vitro* Wound Model

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In individuals with polymicrobial infections, microbes often display synergistic interactions that can enhance their colonization, virulence, or persistence. One of the most prevalent types of polymicrobial infection occurs in chronic wounds, where *Pseudomonas aeruginosa* and *Staphylococcus aureus* are the two most common causes. Although they are the most commonly associated microbial species in wound infections, very little is known about their interspecies relationship. Evidence suggests that *P. aeruginosa*–*S. aureus* coinfections are more virulent than monoculture infection with either species; however, difficulties in growing these two pathogens together *in vitro* have hampered attempts to uncover the mechanisms involved. Here we describe a simple and clinically relevant *in vitro* wound model that supported concomitant growth of *P. aeruginosa* and *S. aureus*. We observed that the ability of *P. aeruginosa* and *S. aureus* to survive antibiotic treatment increased when they were grown together in planktonic cocultures and that antibiotic tolerance was further enhanced when they were grown together in the wound model. We attributed this enhanced tolerance to both the “host-derived” and “bacterium-derived” matrix components. Taken together, our data indicate that *P. aeruginosa* and *S. aureus* may benefit each other by coinfecting wounds and that the host-derived matrix may serve as important a role as the bacterium-derived matrix in protecting bacteria from some antibiotics.

The impact of wound infections on health care is enormous. Infections of the dermis, including burns, surgical-site infections, and nonhealing diabetic foot ulcers, affect approximately 2 million people, cause >200,000 deaths, and account for more than \$18 billion in direct medical costs in the United States annually (1). In fact, chronically infected diabetic foot ulcers are considered the most significant wound care problem in the world (2). The microbial populations of these infected ulcers are typically polymicrobial and biofilm associated and display increased tolerance to antimicrobials (3–6). However, despite the prevalence and severity of these polymicrobial infections, their pathogenesis has been insufficiently studied.

Pseudomonas aeruginosa and *Staphylococcus aureus* are the two most common causes of chronic wound infections and are frequently found together (7–11). Evidence suggests that dual *P. aeruginosa* and *S. aureus* infections are more virulent and/or result in worse patient outcomes than single infections (12–14), and both species are notorious for their resistance to antimicrobials (15–17). Methicillin-resistant *S. aureus* (MRSA) alone accounts for a large portion of hospital-acquired infections (18), and one third of *P. aeruginosa* clinical isolates are resistant to three or more antibiotics, including third-generation cephalosporins and imipenem, which have been the gold standard antibiotics for *P. aeruginosa* infection (19). In addition to the high levels of resistance, *P. aeruginosa* and *S. aureus* also form biofilms *in vivo*, which contribute immensely to antibiotic tolerance. Unlike resistance, which is due to transferable genetic alterations that confer protection against antimicrobials, tolerance implies a transient, nonheritable phenotype (20).

Traditionally, investigations of bacterial pathogens have focused on single-species studies performed under non-physiologically-relevant *in vitro* conditions. However, most chronic wound infections are polymicrobial; members of these communities may display synergistic interactions that can enhance virulence, persistence, or antimicrobial tolerance, and the surrounding environ-

ment may greatly influence these interactions. Difficulties in growing different species of microbes together *in vitro* often make these synergistic interactions challenging to study, and this is certainly true for *S. aureus* and *P. aeruginosa*. Although they are frequently found together in human infections, *P. aeruginosa* quickly kills *S. aureus* when the two are grown together in planktonic cocultures *in vitro* (21, 22). This killing has been attributed to various exoproducts of *P. aeruginosa*, including LasA protease (23), 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO) (24), the *pel* and *psl* products (25), and phenazines such as pyocyanin (26). It is widely accepted that *P. aeruginosa* uses these factors to compete with Gram-positive bacteria for the colonization of different niches (27, 28). However, very little is known about the interactions of *P. aeruginosa* and *S. aureus* in the wound environment. Here we used a convenient and reliable *in vitro* model to study the interactions of *P. aeruginosa* and *S. aureus* in the wound environment and to investigate the role of the host's extracellular matrix in antibiotic tolerance. Our data demonstrate that *P. aeruginosa* and *S. aureus* can exist stably in this *in vitro* wound environment and may mutually benefit from coinfection with regard to antibiotic tolerance. Furthermore, we demonstrate that the presence of the host's extracellular matrix surrounding *P. aeruginosa* and *S. aureus* increases tolerance to some antibiotics.

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MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* strains PAO1 (29) and JM2 (30), the *P. aeruginosa* *algD* deletion strain (31), *S. aureus* strains SA31 (32) and AH1263 (33), and the *S. aureus* *ica* deletion strain (34) have been described previously. Unless otherwise indicated, the wild-type *S. aureus* strain used was SA31.

In vitro wound-like model. The wound-like medium (WLM) was made up of 45% Bolton broth, 50% bovine plasma, and 5% laked horse red blood cells as described previously (35). A 460- μ l volume of WLM was placed in a 5-cm by 0.5-cm glass tube, inoculated with approximately 10^4 to 10^5 CFU of *S. aureus*, *P. aeruginosa*, or both, and grown under static conditions at 37°C for 24 h (unless otherwise indicated).

Planktonic growth. *S. aureus* and/or *P. aeruginosa* was grown in culture flasks, with shaking at 220 rpm, in Luria-Bertani (LB) broth or WLM at 37°C. Samples were taken at the time points indicated in the legends, serially diluted, and plated on *Pseudomonas* isolation agar and/or *Staphylococcus* isolation agar to determine the number of CFU/ml.

Staining and imaging. Imaging was performed on frozen sample sections. Briefly, coagulated WLM was removed from tubes, resized to approximately 1 cm² in diameter, placed in a Tissue-Tek vinyl specimen Cryomold (Sakura Finetek, Torrance, CA, USA) containing a cryomatrix of OCT (optimum-cutting-temperature) compound (Thermo Fisher Scientific, Kalamazoo, MI, USA), and then immediately placed in a freezer at -80°C to allow the OCT compound to solidify. Frozen OCT-embedded samples were sectioned using an OTF5000 cryostat (Bright Instrument Co., Ltd., Huntingdon, Cambridgeshire, England) to a thickness of 4 to 6 μ m and were then directly transferred to Superfrost Plus microscope slides (Thermo Fisher Scientific) and stored at -80°C until ready for visualization. Frozen sample sections were prepared for staining by fixing in 4% paraformaldehyde at room temperature (RT) for 15 min, washed three times in 1× phosphate-buffered saline (PBS), and allowed to air dry for 5 min before the addition of the stain. Matrix components were visualized by staining sections with 50 μ g/ml fluorescein isothiocyanate (FITC)-conjugated concanavalin A (ConA) (Invitrogen, Carlsbad, CA, USA) in the dark for 5 min at RT, washing three times in 1× PBS (5 min each time), and then mounting with ProLong Gold Antifade reagent (Molecular Probes, Eugene, OR, USA) supplemented with 4',6'-diamidino-2-phenylindole (DAPI) to stain DNA. An independent set of frozen sections was subjected to hematoxylin-and-eosin (H&E) staining using standard laboratory techniques and was then mounted with Permount mounting medium (Fisher Scientific) before visualization. Mounted slides were then imaged by fluorescence or light microscopy with an Eclipse 80i microscope (Nikon, Louisville, KY, USA), and images were captured with a DS-Fi1 camera (Nikon) and were analyzed with the NIS Elements program (version 3.00 SP7; Nikon, Japan). For scanning electron microscopy, sections of coagulated WLM were fixed overnight at 4°C in 1.5% glutaraldehyde and 3% paraformaldehyde in 0.1 M Millonig buffer with dextrose. The tissue was then frozen in liquid nitrogen and was fractured with a frozen blade to expose more surface. Tissues were postfixed in 1% osmium tetroxide, taken through a graded series of alcohols, and subjected to critical point drying. The sections were mounted on aluminum stubs using carbon tape and were sputter-coated with gold. Images were acquired with a Hitachi S-570 scanning electron microscope (Hitachi America, Ltd., Brisbane, CA).

Antibiotic tolerance assay. Pellets of planktonic cells, sections of coagulated WLM, or tissue sections from the wounds of infected mice were suspended in 200 μ g/ml gentamicin, 200 μ g/ml ciprofloxacin, 20 μ g/ml tetracycline, or PBS for 5 h. Samples were then suspended in Dey-Engley broth, vortexed, serially diluted, and plated on *Staphylococcus* isolation agar and/or *Pseudomonas* isolation agar to quantitate CFU per milliliter or per gram. It should be noted that Dey-Engley broth is frequently used to neutralize antiseptics in order to avoid false-negative results due to drug carryover; however, to our knowledge, this neutralizing activity does not extend to antibiotics. Here it was used for rinsing. The percentage of cells viable after antibiotic treatment was determined by dividing the number

of cells that survived antibiotic treatment by the number of cells in the PBS control and multiplying by 100.

Mouse wound model. Mice were administered surgical excision wounds as described previously (36) and were infected with 10^5 CFU each of *P. aeruginosa* and *S. aureus*. At the time points indicated in the figure legends, mice were euthanized, and their wound tissues were extracted, weighed, and homogenized in sterile PBS. Serial dilutions of homogenates were used to determine CFU/g of tissue on *Staphylococcus* and/or *Pseudomonas* isolation agar. For tolerance assays, wound tissue was excised and was placed in gentamicin as described above. Mice were housed and studied according to protocols approved by the Institutional Animal Care and Use Committee in the animal facility of Texas Tech University Health Sciences Center (Lubbock, TX).

Statistics. Statistical analyses were performed using GraphPad Prism, version 6, or InStat, version 3. The specific tests used to determine significance are given in the figure legends.

RESULTS

An in vitro "wound-like" model supports concomitant *P. aeruginosa* and *S. aureus* growth. The paucity of studies examining the interspecies relationship between *P. aeruginosa* and *S. aureus* is largely explained by the technical difficulty of growing these two species together in the laboratory. We (and others) have observed that *P. aeruginosa* rapidly kills *S. aureus* when the two are grown together in LB broth, tryptic soy broth, brain heart infusion, and numerous other rich and minimal media (21, 22). Although *P. aeruginosa* and *S. aureus* fail to grow together in planktonic laboratory cultures, they are commonly found together in wound infections. Thus, the first aim of our study was to test the hypothesis that an in vitro "wound-like" environment would support stable concomitant growth of *P. aeruginosa* and *S. aureus* similar to that seen in vivo.

Previously, our collaborators developed a simple and effective method for growing polymicrobial biofilms in vitro (35) using a chopped-meat-based medium supplemented with heparinized plasma and red blood cells. This medium was formulated to represent the conditions of human wounds and contains physiological concentrations of blood components. It has been used by us and others to grow polymicrobial biofilms that accurately reflect the microbial populations of infected human wounds (35, 37, 38). Termed the Lubbock Chronic Wound Biofilm (LCWB), this in vitro model has proved very useful for the rapid screening of different clinical isolates and polymicrobial populations for antimicrobial susceptibility (35, 39). In the LCWB model, bacterial species of interest are inoculated into the WLM in a glass tube and are incubated aerobically, typically for 24 h, at 37°C, under static conditions. Importantly, a sterile pipette tip is placed inside the tube prior to bacterial inoculation and is thought to serve as a surface for biofilm formation (35, 39). However, we noticed that if a coagulase-positive bacterial species (such as coagulase-positive *S. aureus*) was included in the inoculum, the liquid medium coagulated into a jelly-like mass (Fig. 1A) after about 16 h of growth. This is due to the ability of *S. aureus* to activate the coagulation cascade. *S. aureus* secretes staphylocoagulase, which binds to prothrombin, forming a complex called staphylothrombin, which then converts soluble fibrinogen to strands of insoluble fibrin (40). Therefore, for this study, coagulated plasma or the host-derived matrix (HDM), rather than an artificial surface, served as a scaffold to which bacteria could adhere and within which they could reside (Fig. 1). We reasoned that this modification more

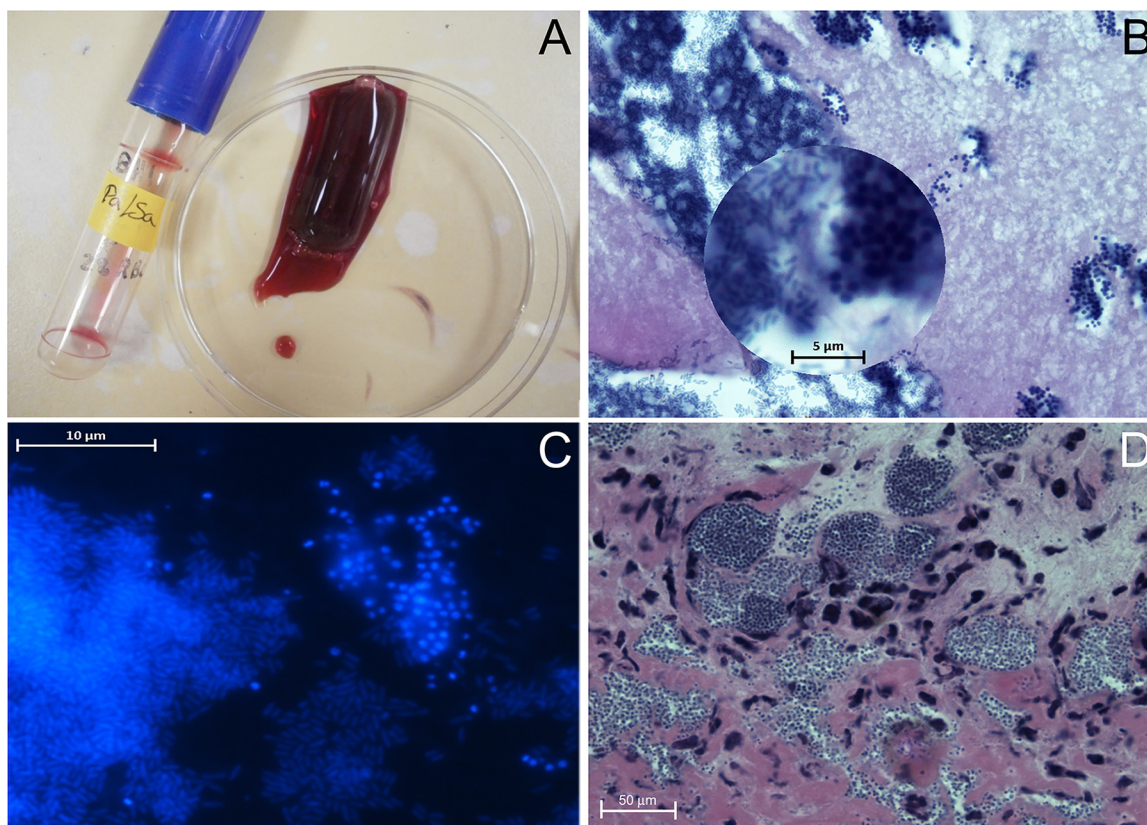


FIG 1 *P. aeruginosa* and *S. aureus* can be grown together in a “wound-like” environment. (A to C) Coagulated WLM supports the growth of *P. aeruginosa* and *S. aureus* (A), which are clearly discernible and in close proximity within the magnified area (B, inset) of a thin section of coagulated WLM stained with H&E (B) or DAPI (C). (D) Clusters of *P. aeruginosa* and *S. aureus* can be visualized in H&E-stained thin sections from mouse wounds. Visualization was carried out with a UPlan FL 40× oil objective (numerical aperture, 1.30).

closely reflected the *in vivo* wound environment and would also help us to better understand the role of the HDM in wound infections. It should be noted (i) that the WLM alone does not coagulate because it is heparinized and (ii) that *P. aeruginosa* will grow in this medium, but it will not coagulate the blood components, because it lacks the enzymes needed to activate the coagulation cascade.

When *P. aeruginosa* and *S. aureus* were cocultured in WLM, discrete clusters of rods and cocci could be seen in close proximity within the HDM (Fig. 1B and C), and the morphology of these clusters, as well as the host matrix surrounding them, was similar to that seen in sections from infected mouse wounds (Fig. 1D) and from human infection sites (3, 41). We compared the growth of *P. aeruginosa*–*S. aureus* cocultures in WLM with that in LB medium. Cocultures were initiated with approximately the same numbers of *S. aureus* and *P. aeruginosa* bacteria (10^4 CFU of each species) and were grown in the same type of culture tubes under static, aerobic conditions at 37°C for 7 days. While the number of *S. aureus* CFU quickly diminished in LB cocultures (Fig. 2A), it remained relatively constant and similar to the number of *P. aeruginosa* CFU in WLM (Fig. 2B). The growth of *P. aeruginosa* and *S. aureus* in WLM more accurately reflected the population kinetics seen in the chronic wounds of mice that were coinfecting with the same numbers and strains of *P. aeruginosa* and *S. aureus* bacteria (Fig. 2C) than when they were cocultured in LB medium. These cultures maintained high counts of viable cells for 7 days, even

though fresh medium was not added. This could indicate that the cells exist in a viable but dormant (not rapidly dividing) state, as in wounds and other chronic infection sites.

The wound environment alters *P. aeruginosa*–*S. aureus* population dynamics. Human wounds are unlikely to be colonized by equal numbers of *P. aeruginosa* and *S. aureus* bacteria at the same time; therefore, we tested how manipulation of the starting bacterial inoculum influenced population dynamics over time. Interestingly, we observed that whether the starting inoculum was high (10^5 CFU/species) (Fig. 3A) or low (10^2 CFU/species) (Fig. 3B), *P. aeruginosa* and *S. aureus* reached a maximum cell density of approximately 10^6 to 10^7 CFU/g after 1 to 2 days, a level similar to the maximum bacterial load seen in mouse wounds (Fig. 2C). The minimum threshold of bacteria needed to initiate a successful coculture was approximately 40 CFU/species. For example, when WLM was inoculated with 40 *S. aureus* CFU and 13 *P. aeruginosa* CFU, no *P. aeruginosa* could be detected after day 2, but *S. aureus* grew to 10^6 CFU/g by day 4 (not shown). We also tested how manipulation of the starting ratio of *P. aeruginosa* to *S. aureus* CFU influenced the population dynamics over time. Whether the starting ratio of *P. aeruginosa* to *S. aureus* CFU was 1:1 (Fig. 3B), 100:1 (Fig. 3C), or 1:100 (Fig. 3D), the two species reached similar maximum cell densities, although when cocultures were initiated with 100 times fewer *S. aureus* than *P. aeruginosa* CFU (Fig. 3C), the number of *S. aureus* CFU remained 1 to 2 log units lower throughout the experiment. It should also be noted that coagula-

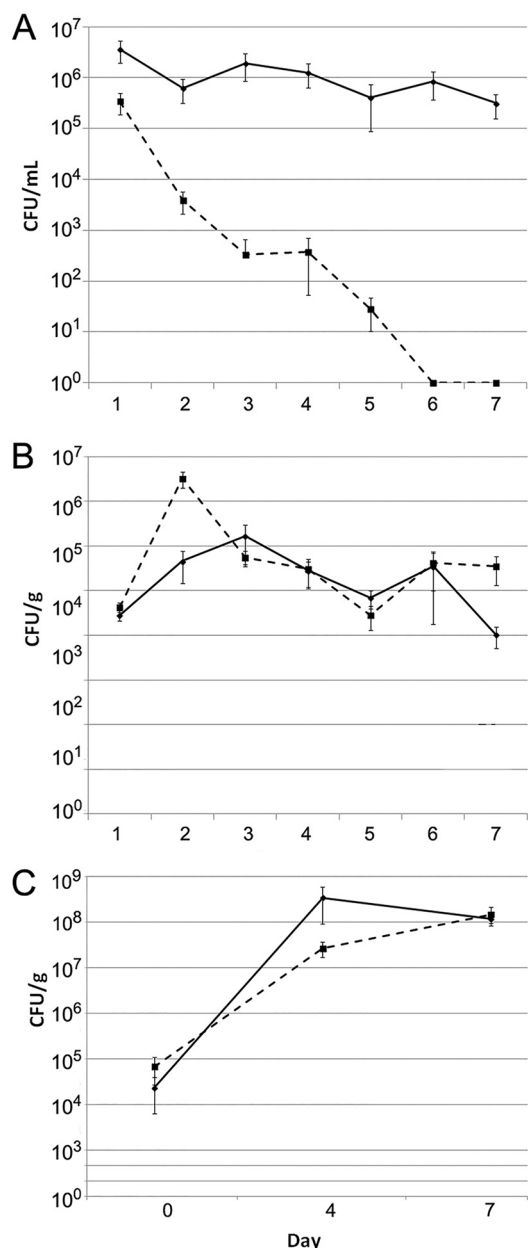


FIG 2 *S. aureus* was quickly eliminated in planktonic LB cocultures but not in static wound environments. (A and B) *S. aureus*–*P. aeruginosa* cocultures were initiated in LB medium (A) or WLM (B) with approximately 10^4 CFU of each species and were grown in glass culture tubes under static, aerobic conditions at 37°C for 7 days. Cultures were sampled at the indicated time points, and the numbers of bacteria were estimated by CFU enumeration on *P. aeruginosa* and *S. aureus* isolation media. Experiments were performed in triplicate. Dashed lines, *S. aureus*; solid lines, *P. aeruginosa*. (C) *S. aureus*–*P. aeruginosa* coinfections were initiated in mouse surgical excision wounds (6 mice per time point) with approximately 10^4 CFU of each species (*P. aeruginosa* and *S. aureus*), and equal numbers were maintained over 7 days.

tion of the WLM was not observed until *S. aureus* numbers exceeded 5×10^4 CFU/mL.

Wounds tend to be colonized by small numbers of resident Gram-positive aerobic cocci, including *S. aureus* and beta-hemolytic streptococci. These species are followed by Gram-negative bacilli, characteristically *P. aeruginosa*, soon afterwards (42).

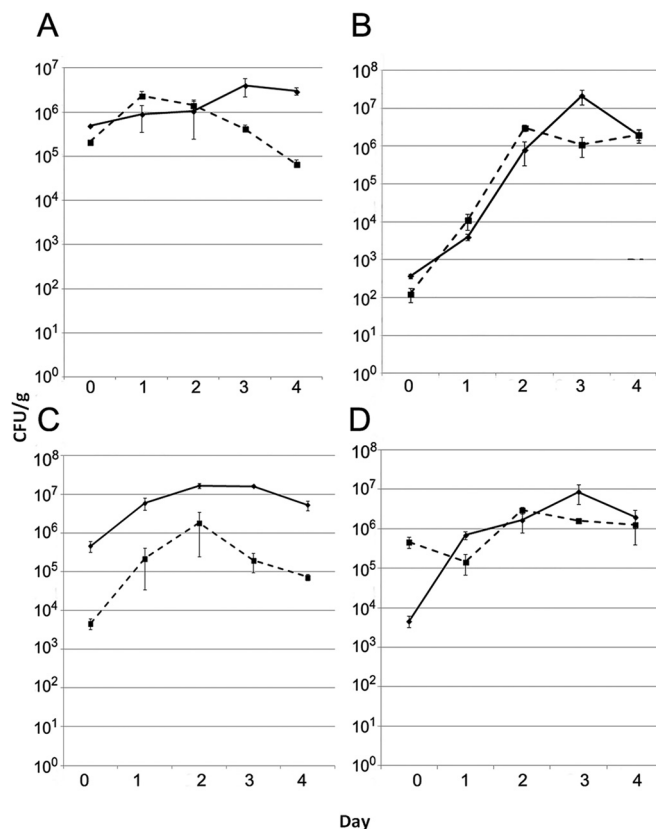


FIG 3 *P. aeruginosa* and *S. aureus* reached equilibrium in the *in vitro* wound environment. Regardless of whether cocultures were initiated in WLM with a high inoculum (10^5 CFU/species) (A, C, and D) or a low inoculum (10^2 CFU/species) (B), or with a *P. aeruginosa*/*S. aureus* starting ratio of 1:1 (B), 100:1 (C), or 1:100 (D), they reached similar maximum bacterial loads after growth in glass culture tubes under static, aerobic conditions at 37°C for 4 days. Cultures were sampled at the indicated time points, and the numbers of bacteria were estimated by CFU enumeration on *P. aeruginosa* and *S. aureus* isolation media. Experiments were performed in triplicate. Dashed lines, *S. aureus*; solid lines, *P. aeruginosa*.

Thus, we tested whether *P. aeruginosa* could colonize secondarily to *S. aureus* in our model. We inoculated approximately 10^4 CFU of *S. aureus* into 460 μ l WLM and then introduced approximately 10^4 CFU of *P. aeruginosa* 2 days later. On day 4 (2 days after the introduction of *P. aeruginosa*), the *S. aureus* level was $3.7 \times 10^6 \pm 7.7 \times 10^5$ CFU/g and the *P. aeruginosa* level was $8.6 \times 10^7 \pm 1.5 \times 10^7$ CFU/g (trimmed mean \pm standard error of the mean [SEM]), indicating successful colonization. Taken together, these data indicate that despite major fluctuations in the starting inocula, *S. aureus* and *P. aeruginosa* reached equilibrium within the *in vitro* wound environment, a finding similar to what was seen *in vivo* and dramatically different from the rapid eradication of *S. aureus* seen in LB medium cocultures. If *S. aureus* and *P. aeruginosa* coinfections truly do result in greater virulence than single infections, then it is important to understand what parameters influence their coexistence. Experiments such as those described above require surveying the population at many different time points and testing several replicates. The use of this *in vitro* wound model makes this type of analysis feasible for investigators without access to animal models.

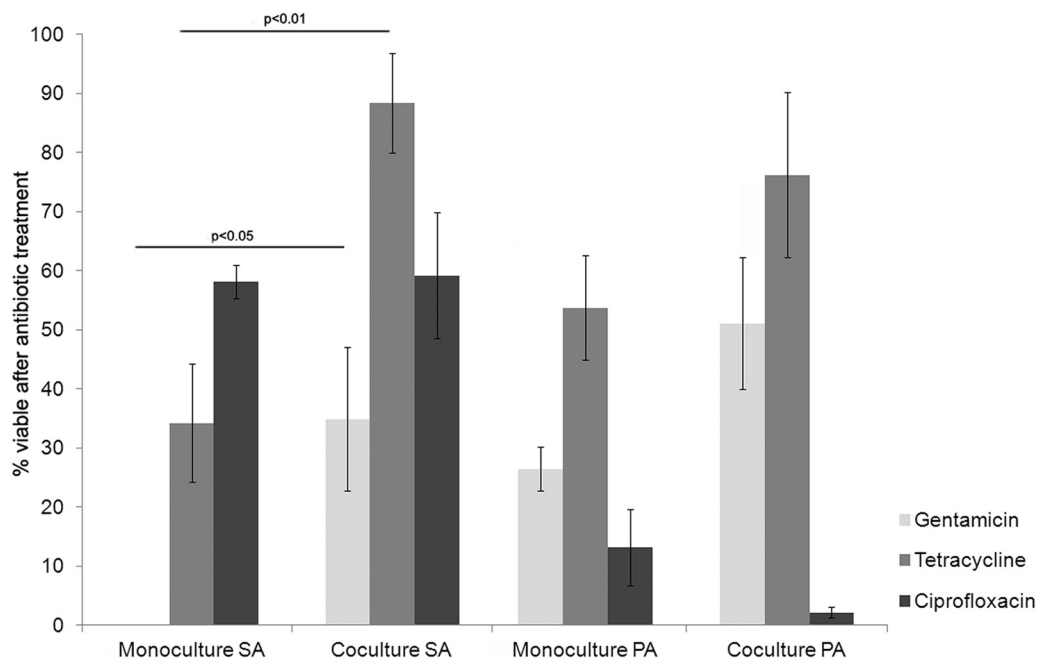


FIG 4 Coculturing *P. aeruginosa* and *S. aureus* altered their antibiotic susceptibilities. Planktonic monocultures or cocultures of *P. aeruginosa* (PA) and *S. aureus* (SA) were grown overnight in WLM, and tolerances to gentamicin, ciprofloxacin, and tetracycline were determined. Cultures were initiated with a ~1:1 starting ratio of *P. aeruginosa* to *S. aureus* and were grown in flasks at 37°C with vigorous shaking, which prevented the coagulation of the medium. One-way ANOVA and the Tukey-Kramer multiple-comparison test were used to test for differences in tolerance between *S. aureus* and *P. aeruginosa* monocultures and cocultures. Each group included at least 6 individual cultures. Error bars represent the standard errors of the means.

***P. aeruginosa*–*S. aureus* cocultures display antibiotic susceptibilities different from those of monocultures in wound-like medium.** After establishing that *S. aureus* and *P. aeruginosa* could coexist in our wound-like model, we sought to determine if coculturing affected their antibiotic susceptibilities. Different combinations of microbes have been reported to exhibit synergism with regard to biofilm-related antibiotic tolerance both *in vitro* (43, 44) and *in vivo* (37). For the most part, changes in biofilm structure and increases in biofilm biomass were thought to account for synergism in these studies. Intuitively, this makes sense, since increased production of extracellular polymeric substances (EPS) would be predicted to inhibit at least some classes of antibiotics. To determine if any potential synergism was dependent on population structure, we first tested whether antimicrobial susceptibility differed between planktonic (i.e., not surface adherent) *P. aeruginosa* or *S. aureus* monocultures and cocultures.

P. aeruginosa and *S. aureus* planktonic monocultures or cocultures were grown overnight in WLM, and antibiotic tolerance was determined as described in Materials and Methods. These cultures were considered planktonic because they were grown with vigorous shaking, which prevented the coagulation of the medium. Cocultures were initiated with a *P. aeruginosa*/*S. aureus* starting ratio of approximately 1:1, and as in coagulated medium, the numbers of *P. aeruginosa* and *S. aureus* bacteria remained relatively equal throughout growth (data not shown). We examined tolerance to antibiotics of three different classes: aminoglycosides (gentamicin), fluoroquinolones (ciprofloxacin), and tetracyclines (tetracycline). As shown in Fig. 4, the tolerance of *S. aureus* to gentamicin increased significantly ($P < 0.05$) from 0% (completely susceptible) to 34.9% ($\pm 12.1\%$) when it was grown in

planktonic coculture with *P. aeruginosa*. A significant increase ($P < 0.01$) in tetracycline tolerance over that of cells in monoculture was also seen for cocultured *S. aureus* cells. Although increases in gentamicin and tetracycline tolerance levels over those of cells in monoculture were also seen for cocultured *P. aeruginosa* cells, these increases were not statistically significant. Notably, the increase in the gentamicin tolerance of *S. aureus* (from 0% in monoculture to 34.9% in coculture) was more than additive (since the gentamicin tolerance of *P. aeruginosa* in monoculture was 18.5%), indicating synergism.

While the specific mechanism of synergism at play here has yet to be defined, it is reasonable to assume that excreted enzymes are involved. *P. aeruginosa* produces several aminoglycoside-modifying enzymes (45), which could have inactivated the gentamicin in the cocultures, thus protecting both species. In addition, Hoffman et al. have demonstrated that the *P. aeruginosa* exoproduct 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO) could protect *S. aureus* from killing by the aminoglycoside tobramycin in *S. aureus*–*P. aeruginosa* cocultures by suppressing *S. aureus* respiration (24). This type of “bystander” protection was also seen in the production of β -lactamases by *P. aeruginosa*, which were thought to protect *S. aureus* from ampicillin treatment in 3-dimensionally (3D) printed *S. aureus*–*P. aeruginosa* bacterial communities (46). Accumulation of environmental DNA (eDNA) in cocultures represents another possibility that could be examined further.

The increase in tetracycline tolerance is harder to explain, because it is thought that the primary mechanisms for tetracycline resistance in *P. aeruginosa* and *S. aureus* are efflux and/or the production of intracellular ribosomal protection proteins (47–49). There is some evidence that the efficacy of tetracycline can differ depending on the growth medium (50). Therefore, it is

possible that medium conditions in the monocultures are more conducive to tetracycline efficacy than those in the cocultures. These differences could perhaps arise from exoproducts that are produced by one species in response to the other or from a specific combination of metabolites that is present only in the cocultures. Taken together, these data indicate that definite changes can be observed in the antibiotic susceptibility patterns of *P. aeruginosa* and *S. aureus* when they are cultured together and that these changes are not dependent on community structure or biofilm formation.

Contributions of the host-derived matrix to antibiotic tolerance. Biofilm studies have traditionally considered only the role of the bacterium-derived matrix or EPS in facilitating antimicrobial tolerance. This is because most biofilm studies have been conducted *in vitro*, where any matrix components present are made by bacteria. However, in the context of wound infections, bacteria are also intimately associated with, and surrounded by, the HDM, which is made up of many of the same components as bacterial EPS (e.g., carbohydrates, DNA, proteins). Therefore, it is possible that the HDM also contributes to antibiotic tolerance. In order to examine this possibility, we compared the tolerance levels of *S. aureus* and *P. aeruginosa* grown planktonically in WLM to their tolerance levels when grown in coagulated WLM, which we considered to be HDM associated. There was a significant increase in the gentamicin tolerance of *S. aureus* residing in HDM over that of *S. aureus* grown planktonically, and coculturing *S. aureus* with *P. aeruginosa* in coagulated WLM potentiated this effect (Fig. 5A). However, the presence of the HDM did not significantly increase the tolerance of *S. aureus* to tetracycline or ciprofloxacin. The gentamicin tolerance of *P. aeruginosa* was also significantly higher when it was cocultured in coagulated WLM with *S. aureus* than when it was cocultured planktonically (Fig. 5B). Unfortunately, because *P. aeruginosa* does not coagulate WLM, we could not compare planktonic and HDM-associated *P. aeruginosa* monocultures.

A classic experiment for testing whether community structure influences antibiotic tolerance is to compare treatment efficacy for an intact community with that for a community that has been physically disrupted (51). Therefore, we next compared the gentamicin tolerance levels of *P. aeruginosa* and *S. aureus* cocultures in intact WLM with those in homogenized coagulated WLM. The number of *P. aeruginosa* cells that survived gentamicin treatment was 8.6-fold lower after the community had been homogenized but was not statistically different from the survival of *P. aeruginosa* from intact communities. However, we saw a 406-fold reduction in the number of *S. aureus* cells that survived gentamicin treatment in homogenized communities ($0.18\% \pm 0.09\%$ tolerance) from that in intact communities ($73.8\% \pm 12.4\%$ tolerance) ($P < 0.001$ by analysis of variance [ANOVA] with the Tukey-Kramer multiple-comparison test). Taken together, these data suggest that the HDM surrounding *S. aureus* and *P. aeruginosa* cells in our model plays a major role in survival of gentamicin treatment. However, the data do not tell us whether or not bacterial EPS also plays a role in this increased tolerance.

Contribution of the bacterial EPS to antibiotic tolerance in the wound environment. It is presumed that *P. aeruginosa*, *S. aureus*, and other microbes reside in biofilms within wounds, because they tend to group together, as with microcolonies seen in biofilms grown *in vitro*, and also because chronic wound infections can be extremely recalcitrant to treatment. However, since

there are no stains or probes that specifically label bacterial EPS but not HDM, it is difficult to demonstrate definitively that bacteria produce EPS within wounds. We have previously used concanavalin A (ConA) staining to image matrix components in sections from infected mouse wounds (32, 36). Although ConA is not specific for bacterial glycocalyx, because it binds to any mannose residues present, we have previously observed marked colocalization of ConA in areas with bacterial aggregates (32, 36). To investigate whether bacteria growing in our *in vitro* wound model produced EPS, we stained thin sections with ConA. As expected, ConA stained the host matrix (Fig. 6A), but there consistently appeared to be areas of more-intense staining surrounding bacterial aggregates (Fig. 6B). Discrete clusters of bacteria were visualized interspersed within the fibrous HDM, presumably between fibrin strands. Scanning electron micrographs of sections of coagulated WLM revealed cocci and rods in close proximity enmeshed in a web of fibrous material (Fig. 6C). However, whether this material was entirely host derived or whether it was also composed of bacterial EPS was not clear.

Several *S. aureus* and *P. aeruginosa* gene products have been implicated in biofilm formation *in vitro*. Therefore, we hypothesized that if bacterial EPS helped facilitate the increased gentamicin tolerance seen in bacteria growing in our model, *P. aeruginosa* or *S. aureus* strains with mutations in key biofilm-related genes may demonstrate reduced levels of tolerance. To investigate the role of *S. aureus* EPS, an *S. aureus* strain with an *ica* deletion, which eliminates its ability to produce the polysaccharide intercellular adhesin (PIA), was used. PIA is composed of repeating units of acetylglucosamine, is the major surface polysaccharide produced by staphylococci, and is known to be upregulated during biofilm infection (52). To investigate the role of *P. aeruginosa* EPS, a *P. aeruginosa* strain with an *algD* deletion was used. Alginate is composed of mannuronic and guluronic acids, is coded for by the alginate operon, and is produced by *P. aeruginosa* *in vivo* (31, 53). In some environments, such as the lung, alginate plays a major role in biofilm formation, although its role in wound infections is less defined.

We first tested whether the deletion of *ica* resulted in lower *S. aureus* tolerance to gentamicin. The *ica* deletion strain and its wild-type parent were grown overnight in our wound model, and gentamicin tolerance was then determined. As shown in Fig. 7A, there was no significant difference in tolerance between wild-type and *Ica*[−] *S. aureus* monocultures. We next tested whether the deletion of *algD* would affect the tolerance of cocultures. When the *P. aeruginosa* *algD* deletion strain was cocultured with wild-type *S. aureus*, we observed reductions in the tolerance levels of both species, but the difference was significant only for *P. aeruginosa* (Fig. 7A). However, in cocultures made up with both mutant strains, both *S. aureus* and *P. aeruginosa* were significantly less tolerant than in wild-type cocultures (Fig. 7A).

Next, we wanted to determine if the reduction in tolerance seen *in vitro* could be recapitulated *in vivo*. For these experiments, mice were given full-thickness surgical wounds as described in Materials and Methods and were infected with either the wild-type or the mutant strains of *P. aeruginosa* and *S. aureus*. After 4 days of infection, the mice were euthanized, and wound tissue was harvested. The bacterial loads of *P. aeruginosa* and *S. aureus* in the tissue were determined, and gentamicin tolerance assays were performed. As shown in Fig. 7B, although the coinfections resulted in similar bacterial loads within the wounds,

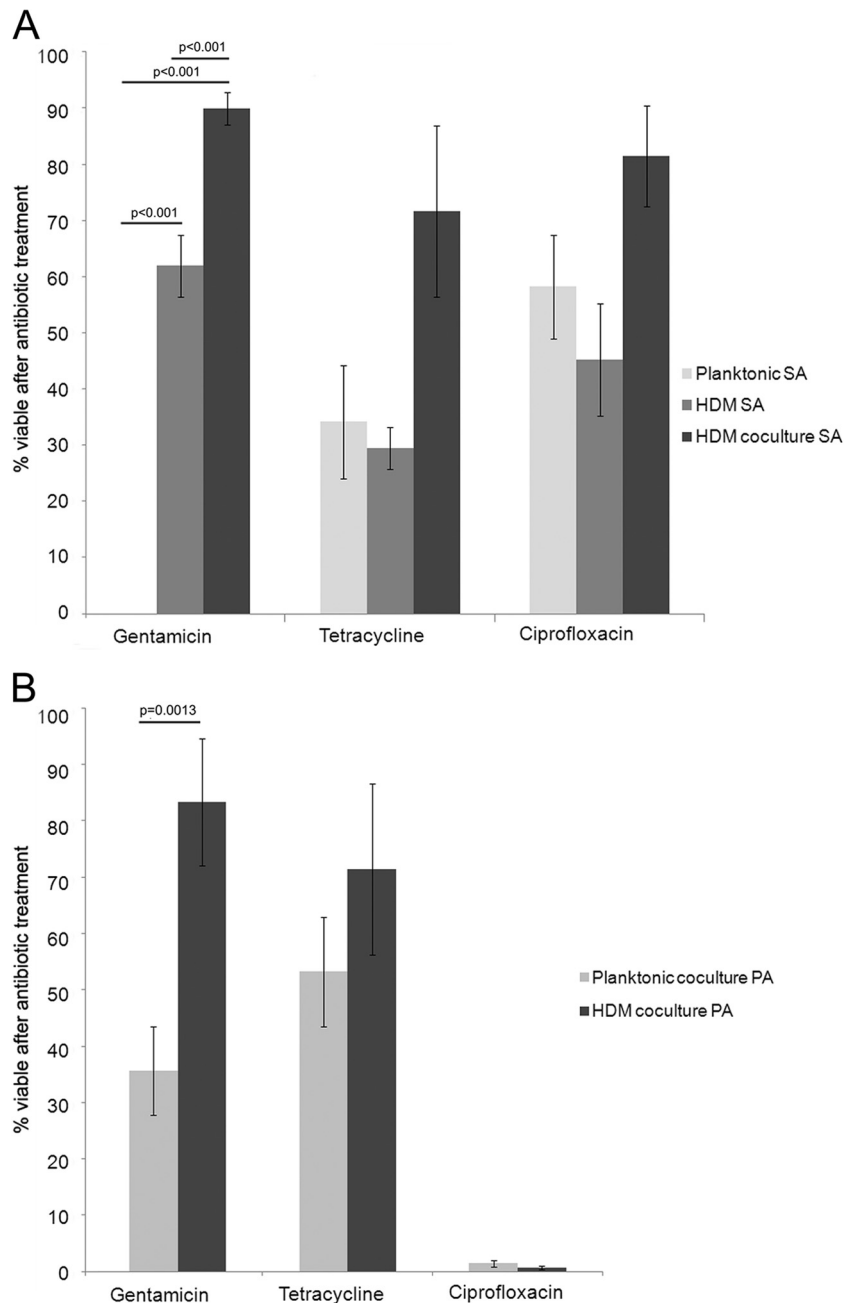


FIG 5 The host-derived matrix contributed to the gentamicin tolerance of *S. aureus* (A) and *P. aeruginosa* (B). *S. aureus* monocultures and *S. aureus*–*P. aeruginosa* cocultures were grown aerobically overnight at 37°C either in culture tubes under static conditions, which allows for the coagulation of the WLM, or in flasks with vigorous shaking, which inhibits coagulation and results in a planktonic population. The gentamicin, tetracycline, and ciprofloxacin tolerances of samples from these cultures were then measured. One-way ANOVA and the Tukey-Kramer multiple-comparison test were used to test for differences in tolerance to each antibiotic group between *S. aureus* cultures. Two-tailed, unpaired *t* tests were used to test for differences in tolerance to each antibiotic group between planktonic and HDM-associated *P. aeruginosa* cocultures. Each group included at least 6 individual cultures. Error bars represent the standard errors of the means.

the gentamicin tolerance levels of the mutant strains were significantly reduced, a finding similar to our *in vitro* results. Taken together, these results indicate that bacterial EPS does contribute to gentamicin tolerance, both in our *in vitro* model and in actual wounds. The deletion of *algD* influenced the tolerance of *P. aeruginosa* even when it was cocultured with wild-type *S. aureus*, suggesting that even though other extracellular polymeric sub-

stances are produced by *P. aeruginosa*, alginate is a key EPS component in the wound environment. It should be noted that we compared the gentamicin tolerance levels of the wild-type *P. aeruginosa* strain and the *algD* mutant grown planktonically and found them comparable (data not shown). We found it interesting that an *ica* deletion did not affect the tolerance of an *S. aureus* monoculture but did contribute to the significantly reduced tol-

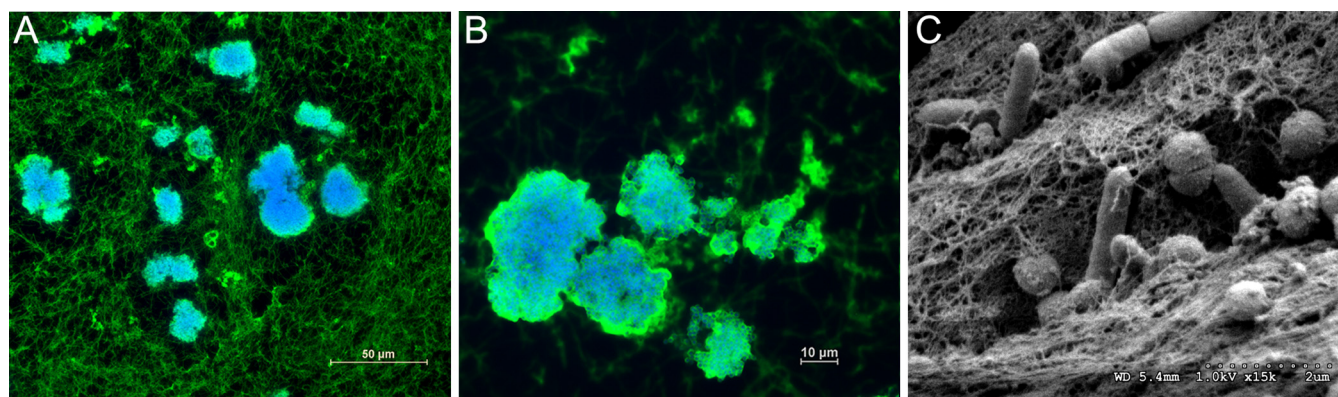


FIG 6 Imaging of *P. aeruginosa* and *S. aureus* in the HDM. (A and B) One-day-old *P. aeruginosa*–*S. aureus* cocultures in coagulated WLM were frozen in a cryomatrix and were sectioned. Sections were stained with FITC-conjugated ConA and DAPI and were visualized by fluorescence microscopy (with UPlan FL 40× [A] and 100× [B] oil objectives [numerical aperture, 1.30]). These images revealed discrete clusters of bacteria, which stained intensely for ConA, interspersed in the fibrous host matrix. (C) Scanning electron microscopy (magnification, ×15,000), performed on glutaraldehyde- and paraformaldehyde-fixed sections from 1-day-old *P. aeruginosa*–*S. aureus* cocultures in coagulated WLM, revealed cocci and rods in close proximity enmeshed in a web of fibrous material.

erance of *S. aureus* in coculture with the *P. aeruginosa* *algD* deletion strain. At this time, we are not sure why the *ica* deletion affected tolerance only when *S. aureus* was cocultured with the *P. aeruginosa* *algD* deletion strain.

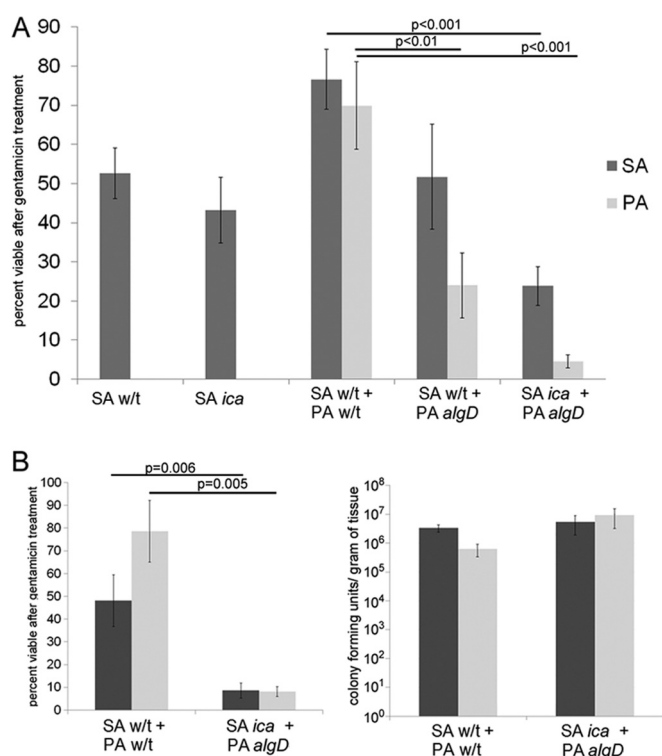


FIG 7 *P. aeruginosa* and *S. aureus* biofilm mutants demonstrated reduced levels of tolerance in the wound environment. The gentamicin tolerance levels of wild-type (w/t) *S. aureus* and *P. aeruginosa* were compared to those of isogenic mutants with mutations in EPS-related genes. Strains were grown in coagulated WLM (A) or in murine surgical excision wounds (B). (A) One-way ANOVA and the Tukey-Kramer multiple-comparison test were used to test for differences in tolerance among strains grown *in vitro*. Each group included at least 6 individual cultures. Error bars represent the standard errors of the means. (B) A paired *t* test was used to test for significant differences in tolerance between *P. aeruginosa* or *S. aureus* strains grown *in vivo* (6 mice per group; error bars represent the standard errors of the means).

DISCUSSION

Although it is known that environment greatly influences the behavior of bacteria, most studies are still performed on planktonic cultures in rich media. And although many infections are polymicrobial, most studies are still performed with a single species. We chose to study interactions between *S. aureus* and *P. aeruginosa* because these two species are the first and second most common causes of chronic wound infections and because they are the two species most commonly found together in polymicrobial wound infections (7–11, 15–17). While there is some evidence that *P. aeruginosa* and *S. aureus* cause more-severe infections together than alone (12–14), difficulties in growing these two microbes together *in vitro* have hampered progress in understanding their interspecies interactions.

In our study, *P. aeruginosa* and *S. aureus* clearly interacted differently in the wound-like environment than in traditional laboratory growth media. While *S. aureus* was quickly eradicated when cocultured with *P. aeruginosa* in LB medium and several other types of media, the two species coexisted in WLM. We initially thought this was due to the localization of *P. aeruginosa* and *S. aureus* in the HDM, e.g., that the two species were not in close enough contact for the staphylolytic exoenzymes of *P. aeruginosa* to affect *S. aureus*, or that adhesion to the HDM protected *S. aureus* from *P. aeruginosa*. However, *P. aeruginosa* and *S. aureus* were also successfully cocultured planktonically in WLM. In these cultures, coagulation of the plasma was prevented by vigorous shaking, and still *S. aureus* persisted. This indicated that the WLM had some effect on one or both of these species that promoted the survival of *S. aureus*. This does not mean that the spatial localization of *P. aeruginosa* and *S. aureus* is not responsible for coinfections *in vivo* but simply that other mechanisms may also be at play. These additional mechanisms are under investigation in our laboratory.

While there are many ways in which bacterial species may display synergism, we chose to investigate synergism with regard to antimicrobial tolerance. However, this model could also be used to investigate synergism in regard to other phenotypic characteristics, e.g., the production of virulence factors. Here we wanted to examine separately the effect of coculturing and the effect of the presence of the HDM on tolerance. So we separated these two

variables by measuring the tolerance levels of *P. aeruginosa* and *S. aureus* alone and in coculture under planktonic and HDM-associated conditions. We found that coculturing increased the tolerance of *S. aureus* to gentamicin and tetracycline but did not affect its ciprofloxacin tolerance. Very few studies have examined the effects of polymicrobial interactions on antibiotic susceptibility. However, if some species of bacteria can alter the antibiotic susceptibility of others, this would be important to know, since most wound infections are polymicrobial. Instead, conventional susceptibility testing involves isolating members of the population and testing the susceptibility of planktonic pure cultures. In fact, a study published in 1969 included the “surprising finding” that “the combination of two sensitive organisms could give a resistant result” (54). Instead of pointing out the potential clinical importance of these findings with regard to the treatment of mixed-species infections, the authors argued that these confusing results should be avoided by always performing susceptibility testing on pure cultures. We would argue that the cumulative susceptibility of the entire microbial population should be considered when one is determining the most effective drugs to use. This approach has been adopted recently by our clinical collaborators. Using their LCWB model, the Wolcott group regularly reconstitutes the microbial population from the wounds of individual patients by inoculating WLM with debridement samples. They then perform susceptibility testing on the entire microbial population to determine what combination of drugs is most efficacious (35, 55; R. D. Wolcott, personal communication). While preliminary indications appear promising, the efficacy of susceptibility testing on communities to improve antibiotic treatment has not been tested in a controlled trial.

This study also aimed to determine if the HDM influenced antibiotic tolerance. The first critical step in the microbial colonization of host tissue is the adherence of bacteria to constituents of the host extracellular matrix, including fibronectin, laminin, and collagen (56, 57). While many *in vitro* biofilm models have utilized host extracellular matrix factor-coated surfaces (e.g., collagen-coated glass slides and collagen encapsulation to simulate biofilm) (58, 59), the microbial biofilm dogma has historically considered only the role of the bacterium-derived matrix or EPS in antimicrobial tolerance. We found that being embedded in a matrix clearly protected *S. aureus* and *P. aeruginosa* from gentamicin. Curiously, the HDM did not significantly influence the penetration and/or efficacy of the other antibiotics tested. Penetrating ability is thought to rely to a great extent on charge. Because the overall net charge of the EPS is negative, negatively charged compounds should penetrate more readily than those that are positively charged. This is also true for the similarly composed HDM, where basophilic connective tissue poses a challenge for the penetration of topical antibiotics. Recently, Tseng et al. demonstrated that the positively charged antibiotic tobramycin became sequestered in the peripheries of biofilms, while the neutral antibiotic ciprofloxacin readily penetrated them (60). Similarly, we observed that the efficacy of ciprofloxacin was not influenced by the HDM but that the positively charged antibiotic gentamicin demonstrated poorer efficacy in coagulated WLM. Tetracycline is a mixture of zwitterions, cations, and anions, the proportions of which depend on the pH of the environment. The efficacy of tetracycline also was not significantly affected by the HDM in our study, which could mean that its overall net charge in this particular environment is neutral.

We observed more-intense ConA staining in areas with bacterial clusters, which suggested that the bacteria in the HDM also produced their own EPS. Our *in vitro* and *in vivo* experiments with *S. aureus* and *P. aeruginosa* EPS mutants confirmed that bacterium-derived EPS components do contribute to increased gentamicin tolerance in wound environments. Strikingly, when these key EPS-related genes were deleted in both species, the coinfection strains were extremely susceptible to gentamicin. We compared ConA-stained sections from wild-type cocultures with those from cocultures of strains with *ica* and *algD* deletions, and we did not see a dramatic difference in the intensity of staining. Therefore, we do not at this time have evidence that the striking reduction in tolerance is due to a major reduction in the biomass of the EPS. It is possible that physical interactions between the different *S. aureus* and *P. aeruginosa* EPS constituents increase the structural integrity of the biofilm or modulate the charge of the EPS, which helps to protect bacteria. While the precise mechanisms are not clear at this time, it is apparent that there is still much to be understood about the interactions of these two species in wounds.

In summary, we assert that this *in vitro* wound-like model is a convenient, inexpensive, and reliable way to grow multiple species of bacteria together and study them in a physiologically relevant environment. While there are definite limitations to the model, e.g., the lack of immune cells, it is a significant improvement over conventional single-species planktonic growth in standard culture media and provides a fair representation of the wound environment. In this model, *S. aureus* and *P. aeruginosa* displayed synergism with regard to antibiotic tolerance, which was enhanced both by the surrounding HDM and by their own EPS. Taken together, these data suggest that growing together in wounds may provide mutual benefit to *P. aeruginosa* and *S. aureus*.

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